

Article

Producing Natural Flavours from Isoamyl Alcohol and Fusel Oil by Using Immobilised *Rhizopus oryzae* Lipase

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Abstract: Enzymatic synthesis of short-chain esters (flavours) might enable their labelling as natural, increasing their value. Covalently immobilised *Rhizopus oryzae* lipase (EO-proROL) was used to synthesise isoamyl butyrate and acetate. In cyclohexane, the best performer reaction solvent, 1.8 times higher yield of isoamyl butyrate (ca. 100%) than isoamyl acetate (ca. 55%) was obtained. Optimum initial acid concentration (410 mM) and acid:alcohol mole ratio (0.5) were established by a central composite rotatable design to maximise isoamyl butyrate single-batch and cumulative production with reused enzyme. These conditions were used to scale up the esterification (150 mL) and to assess yield, initial esterification rate, productivity and enzyme operational stability. Commercial isoamyl alcohol and fusel oil results were found to be similar as regards yield (91% vs. 84%), initial reaction rate (5.4 $\mu\text{M min}^{-1}$ with both substrates), operational stability (40% activity loss after five runs with both) and productivity (31.09 vs. 28.7 mM h^{-1}). EO-proROL specificity for the structural isomers of isoamyl alcohol was also evaluated. Thus, a successful biocatalyst and product conditions ready to be used for isoamyl ester industrial production are here proposed.

Keywords: *Rhizopus oryzae* lipase; flavor; fusel oil; short-chain esters; esterification; *Komagataella phaffii*; immobilization; isoamyl alcohol; specificity



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1. Introduction

Flavour esters are short-chain esters commonly found in various fruits and plants that possess favourable sensory attributes (floral, spicy, fruity) and are used by the food, beverage, cosmetics and pharmaceutical industry, among others [1,2]. The flavour and fragrance market was valued at \$28 billion in 2019 and is expected to expand at a compound annual growth rate (CAGR) of 4.7% to \$35 billion from 2021 to 2027 [3].

Currently, flavour esters are primarily obtained by extraction from natural sources. The process involves extensive downstream work, owing to the typically low concentrations at which these compounds are present in the environment. Also, using natural sources, such as plants and flowers, introduces uncontrolled variables like agricultural and climate conditions into the process [1,4]. This has raised the need for the production of flavours (chemically or enzymatically) in order to meet the increasing demand for short-chain esters [5]. The typically poor selectivity of chemically catalysed processes can result in unwanted side reactions yielding racemic mixtures, rather than pure substances, and even by-products. Besides, the processes usually require harsh conditions that are scarcely compatible with Green Chemistry, a positive industrial trend to face the present environmental crisis and comply with increasingly stringent public policies [6–8]. Also, European Legislation (EC 1334/2008, EC 32/2009) precludes labelling chemically produced esters as natural, which has placed them at a loss on the growing market for natural products [9].

Lipases, which are glycerol ester hydrolases (E.C.3.1.1.3), have lately emerged as potential alternative choices. These enzymes can catalyse a wide variety of synthetic reactions in non-aqueous media, for instance, esterification and transesterification [10], which have been extensively used to obtain a variety of products, ranging from biodiesel through structured lipids to short-chain esters (flavours) [11,12]. Enzymes can help to circumvent the problems encountered in the chemical synthesis and extraction of esters. Thus, their high activity, selectivity and specificity allow the synthesis of flavour molecules of a high quality and purity, the use of milder reaction conditions, which facilitates preservation of thermolabile molecules and reduces energy consumption, allows easier downstream processing of the end-product and, ultimately, the development of greener bioprocesses with less adverse impacts on the environment. In addition, enzymatically obtained synthetic substances, complying with legislation on natural products, possess a substantially increased market value [1,13,14].

Rhizopus oryzae lipase (ROL) is being increasingly used in bioprocesses on the grounds of its thermostability and tolerance of organic solvents, two positive traits for industrial use [13,15,16]. Also, some industrially relevant features of ROL have been further improved by modifying its glycosylation patterns, or its aminoacidic sequence (especially its pro-sequence), for increased stability [17–20]. For industrial use, however, the enzyme is preferably immobilised onto a suitable support in order to ensure safe production of the ensuing food additives, facilitate reuse, avoid aggregation and autolysis of the enzyme, and increase its stability, provide flexibility in reactor design and cost-effectiveness [21–24]. Heterologous synthesis of enzymes has also enabled more inexpensive production of biocatalysts in well-established cell factories [25]. Specifically, ROL has been successfully produced in various hosts, but especially well in the methylotrophic yeast *Komagataella phaffii* (*Pichia pastoris*). This yeast contains no endogenous esterases or lipases, and can secrete heterologous proteins, enact eukaryotic post-translational modifications and grow at high cell densities (ca. 100 g L^{−1} dry cell weight) in defined media [26–29].

Rhizopus oryzae lipase has previously been used to obtain short-chain natural esters, such as ethyl butyrate, butyl acetate and octyl acetate, by esterification or transesterification [13]. Using high acid concentrations for esterification detracts from enzyme performance [30]; by contrast, transesterification replaces acids with esters and is less prone to deactivating the enzyme [31]. However, transesterification reactions usually involve non-natural reactants, so esterification is to be preferred if the natural flavour market is targeted. Regarding alcohols, branched ones have been found to exert steric hindrance on the conversion into esters [32]. As a result, the enzymatic synthesis of isoamyl esters, such as isoamyl acetate (banana flavour) [31] and isoamyl butyrate (pear, apricot and banana flavours) [33], has become a widely adopted research model and revealed the feasibility of using fusel oil (a by-product of ethanolic fermentation) as an alternative alcoholic substrate to significantly reduce costs and comply with the principles of circular economy [34,35].

In this work, recombinant *Rhizopus oryzae* lipase, with 28 C-terminal amino acids of its pro-sequence fused to the N-terminal mature sequence (proROL), was covalently immobilised onto polymethacrylate supports (Purolite®) containing surface epoxy and octadecyl groups (EO-proROL). The biocatalyst was used to obtain isoamyl butyrate and isoamyl acetate by esterification. The influence of the solvent was evaluated, and single-batch and cumulative production were maximised by optimising the initial acid concentration and acid:alcohol mole *ratio* with a central composite design in combination with response surface methodology. The optimum conditions thus found were used to scale up the reaction and the results thus obtained were compared with those of esterifying butyric acid with waste from alcoholic fermentation (fusel oil), as a cheap source of isoamyl alcohol. The specificity of the biocatalyst for structural isomers of the alcohol was also assessed with both alcoholic substrates.

2. Results and Discussion

2.1. Reaction Solvent for Butyric and Acetic Acid Esterification with Isoamyl Alcohol

The solvents initially used as reaction media in the esterification of butyric and acetic acid with isoamyl alcohol were cyclohexane and hexane, both of which are allowed for the production of foodstuffs and food ingredients by European legislation (2009/32/EC). Other allowed solvents were avoided because they have a strong odour, low concentrations are allowed after purification or because they might have interfered with the reaction.

The two solvents chosen were assessed by following the procedure described in Section 3.5. Higher concentrations of acid and isoamyl alcohol were not tested to avoid potential biocatalyst deactivation, as previously described [2]. Under the established conditions, butyric acid esterification with isoamyl alcohol reached yields close to 100% and approximately 1.8 times higher than acetic acid esterification (Figure 1), despite the longer reaction time used in the latter (5 h vs. 24 h). In fact, this result is logically aligned with previous reports, in which acetic acid was described as a more potent enzyme inhibitor than other acids, such as propionic or butyric acids. This is because acetic acid causes more severe dead-end inhibition through preferential reaction with the serine residue at the active site of the lipase [36,37].

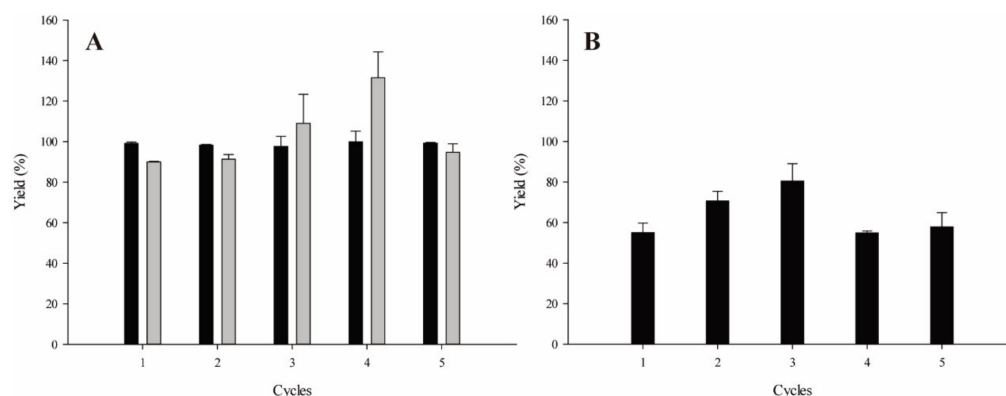


Figure 1. Esterification yield (%) of butyric acid (A) and acetic acid (B) with isoamyl alcohol in cyclohexane (black) and hexane (grey). The isoamyl butyrate synthesis reaction was extended for 5 h and the isoamyl acetate synthesis reaction for 24 h.

Interestingly, EO-proROL (see Section 3.4) showed excellent esterification performance with isoamyl alcohol, even though esterification of β - and γ -branched alcohols is thought to sterically hinder enzyme activity [32,38]. Although whether a linear or branched alcohol is used is seemingly not a crucial factor, according to some reports [39], some authors have suggested that whether it is primary or secondary does influence reaction performance [40].

As can be seen from Figure 1, EO-proROL exhibited a high operational stability. Thus, no loss of enzyme activity was detected after 5 reaction cycles of isoamyl butyrate synthesis with either cyclohexane or hexane as the solvent—the yield was ca. 100%. The increased yield variability observed in hexane (more than 20%) was probably due to evaporation, especially over the long reaction time used to synthesize isoamyl acetate. Consequently, to prevent the real concentration of the substrates from being altered, due to the evaporation of the reaction solvent and having a negative effect on the biocatalyst, cyclohexane was chosen as the solvent for the following studies. Isoamyl acetate biosynthesis was also discarded, owing to the low reaction yields obtained under the studied conditions.

2.2. Experimental Design: Optimisation of Isoamyl Butyrate Single-Batch and Cumulative Production

The DoE methodology is extensively used to assess the individual and combined effects of operational variables on one or several responses [41,42] in processes such as flavour ester biosynthesis [43,44]. In this work, we used it to understand the effect of, and relationship between, two major variables (viz., acid concentration and acid:alcohol mole

ratio) in order to maximise single-batch and cumulative production of isoamyl butyrate in cyclohexane with a Box-Hunter design. The resulting matrix (Figure 2) consisted of 11 experiments, the data of which were fitted to Equation (4) (see Section 3.5.1), and the fitted data being used to construct the response surfaces of Figure 3 for easier understanding. The values of the coefficients are listed in Table 1. The statistical significance of the different functions, and their respective coefficients, were assessed via ANOVA (Table 1).

Table 1. Box-Hunter design, independent variables, response values obtained and results of the ANOVA analysis.

Box-Hunter Design					
Experimental Run	Variables			Production Response	
	[Butyrate] (mM)	Acid:Alcohol Mole Ratio	[Isoamyl Alcohol] * (mM)	Single-Batch (μmol)	Cumulative (μmol)
1	118.37	1.78	66.49	105.2	537.14
2	10	1.25	8	12.7	78.74
3	641.63	0.72	891.56	194.6	968.8
4	380	1.25	304	241.2	1092.4
5	380	2	190	200.6	686.9
6	380	0.5	760	263.2	1293.2
7	750	1.25	600	75.8	383.7
8	118.37	0.72	164.48	153.9	742.7
9	380	1.25	304	263.1	972.9
10	641.63	1.78	360.40	199.6	672
11	380	1.25	304	281.4	1006.14

Statistical Analysis					
Production	F Test p-Value	LOF Test p-Value	R ²	Adjusted-R ²	Predicted-R ²
Single-batch	<0.01	0.3593	0.9193	0.8991	0.8566
Cumulative	<0.01	0.3085	0.9541	0.9345	0.8620

Model		Single-Batch Production		Cumulative Production	
Parameters	Coefficient	p-value		Coefficient	p-value
β ₀	260	<0.05		1036	<0.05
β ₁	28	0.02		99	0.016
β ₂	NS	>0.05		−170	<0.01
β ₁₂	NS	>0.05		NS	>0.05
β ₁₁	104	<0.01		−370	<0.01
β ₂₂	NS	>0.05		NS	>0.05

* As calculated from the butyric acid concentration and acid:alcohol mole ratio. NS: not statistically significant.

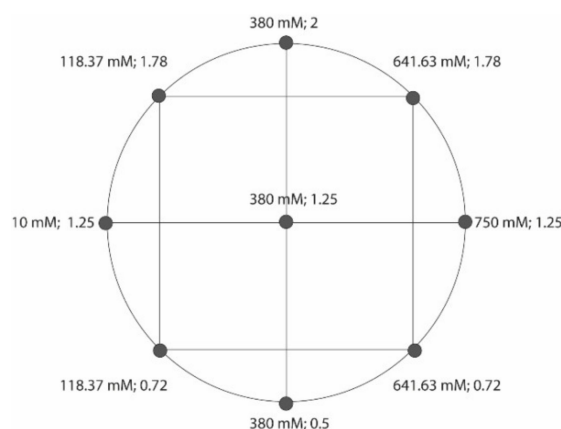


Figure 2. Box-Hunter design matrix representing the butyric acid concentration and acid:alcohol molar ratio used to maximise single-batch and cumulative production.

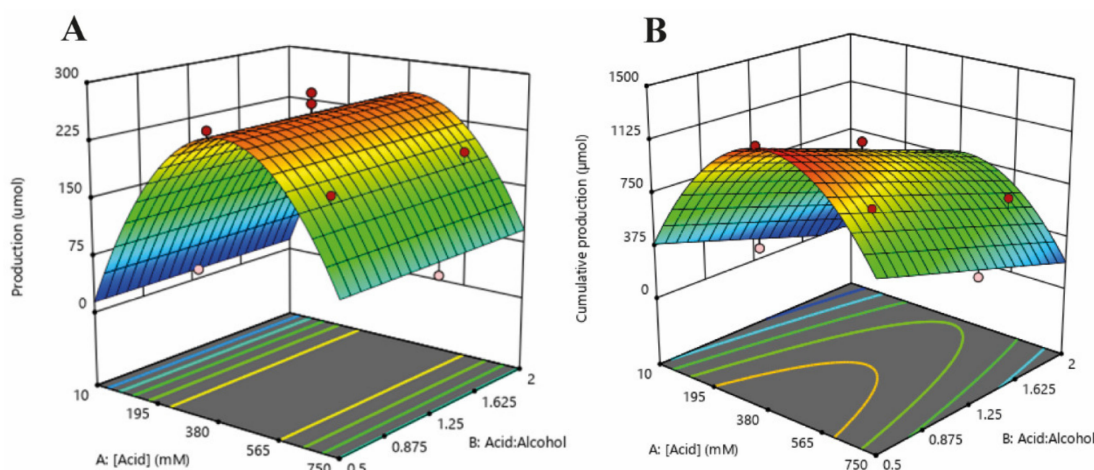


Figure 3. Experimental response surfaces for single-batch (A) and cumulative production (B) of isoamyl butyrate at different initial butyric acid concentrations and acid:alcohol mole ratios. The red dots correspond to the design points listed in Table 1. Measured values greater and smaller than the predictions are shown in dark red and light red, respectively.

The experimental data for the first cycle of isoamyl butyrate production were fitted to Equation (4) and the coefficients of the terms β_2 , β_{12} and β_{22} were found not to be statistically significant (p -value > 0.05), resulting in the Equation (1), a quadratic function of the acid concentration alone. R^2 for the function exceeded 0.9 and the difference between adjusted- R^2 and predicted- R^2 was less than 0.2, so the goodness of fit was acceptable.

$$\text{Production } (\mu\text{mol}) = 260 + 28 \times [\text{acid}] - 104 \times [\text{acid}]^2 \quad (1)$$

Interestingly, the acid:alcohol mole *ratio* was scarcely influential on ester production. This result is similar to that of a previous study [45], but contradicts one where the influence of the initial acid concentration on isoamyl butyrate yield was not considered as a DoE variable [33]. Therefore, only the initial acid concentration influenced production of the ester here, production peaking with a concentration around 400 mM (Figure 3A, Table 1) and declining above that concentration. Previous studies with ROL found butyrate concentrations of 54.6 mM [46] and 225 mM [30] to result in optimal esterification and production of ethyl butyrate. The fact that production decreased above 400 mM butyric acid here could have been caused by deactivation or inhibition of the enzyme. In fact, high acid concentrations were previously found to promote denaturation of immobilised enzymes and to render them inactive as a result [47]. Also, as previously noted for acetic acid, butyric acid can, to a lesser extent, bind to the acyl-enzyme complex unproductively and give a dead-end intermediate unable to form an ester [48]. In addition, esterification reactions have been shown to follow the ping-pong kinetic model and may, thus, be subject to acid-mediated inhibition [49]. However, some studies have shown esterification to be hindered by alcohols, and also by both alcohols and acids [50,51].

Evaluating cumulative production allowed us not only to identify the most productive conditions after 5 reaction cycles, but also to assess, indirectly, the best conditions for retaining biocatalyst activity correlated with the operational stability. Experimental data for cumulative production fitted Equation (4), coefficients β_{12} and β_{22} had p -value > 0.05 and were thus deemed non-significant, so Equation (2) was obtained with $R^2 > 0.95$, and adjusted- R^2 and predicted- R^2 values differing by less than 0.2.

$$\text{Cumulative production } (\mu\text{mol}) = 1036 + 99 \times [\text{acid}] - 170 \times \text{acid:alcohol} - 370.2 \times [\text{acid}]^2 \quad (2)$$

As expected, increasing the initial acid concentration to about 400 mM resulted in increasing cumulative production, higher acid levels leading to a decline, however (Figure 3B, Table 1). This was a result of increased acid concentrations detracting from operational sta-

bility in the biocatalyst for the above-described reasons. Raising the proportion of alcohol (i.e., lowering the acid:alcohol mole *ratio*) increased cumulative production, in line with previous results and consistent with the protective role of the alcohol [52,53]. Although a high alcohol concentration in the reaction medium can also have a negative impact on enzyme performance, none of the concentrations used here fell above that theoretical threshold (Figure 3B). The negative impact of short-chain alcohols on esterification has been ascribed to their sequestering water molecules needed to maintain the 3D conformation and activity of the lipase [54,55].

Based on the previous results, an initial butyric acid concentration of 410 mM and an acid:alcohol mole *ratio* of 0.5 were chosen as optimal to maximise single-batch and cumulative ester production. The predicted maximum values (256.28 and 1282.78 μmol , respectively) were experimentally validated—the highest deviation was only about 2.5%.

2.3. Reaction Scale Up: Isoamyl Butyrate Production, Fusel Oil Use and Half-Lives

The previously established optimum butyric acid concentration and acid:alcohol mole *ratio* were used to scale up the esterification reaction to a laboratory bioreactor as described in Section 3.5.2. In this section, a bioreactor with mechanical stirring was used, which more adequately represents the reactors used at industrial level compared to the orbital agitation used for the screening of the optimal reaction conditions. Commercial isoamyl alcohol and fusel oil (chiefly containing isoamyl alcohol) were used as substrates to assess single-batch yield, initial esterification rate, productivity and enzyme operational stability. The high moisture content of fusel oil required drying over 3 Å molecular sieves for 48 h [56] and centrifugation to remove any solid residue prior to use. The proportion of isoamyl alcohol concentration in dry fusel oil, as assessed by GC analysis, was 92%, and other alcohols, such as ethanol and pentanol, were also found [34].

Figure 4A shows the esterification yield obtained with commercial isoamyl alcohol as substrate, which reached 91% after 720 min. The fact that the yield obtained after 5 h was similar to that previously found in 15 mL tubes confirmed that the reaction was successfully scaled up. As can be seen from Figure 4B, the esterification yield with isoamyl alcohol contained in fusel oil was similar to that obtained with commercial isoamyl alcohol. As expected, the reaction with fusel oil gave a lower isoamyl butyrate yield after 720 min reaction (84% instead of 91%), owing to the presence of other alcohols also acting as substrates and, hence, using some butyric acid to yield other flavours, like ethyl butyrate or pentyl butyrate. In fact, purification processes like distillation would be necessary to extract pure isoamyl butyrate from the reaction mixture with fusel oil, as previously reported for purifying alcohols in this substrate [34]. Productivity in the first reaction batch was similar with commercial isoamyl alcohol and isoamyl alcohol contained in fusel oil (31.09 vs. 28.7 mM h^{-1}). These productivities are lower than, although of the same order of magnitude to, those previously obtained with Lipzyme TL IM (55 mM h^{-1}) [45] and *Rhizopus* sp. lipase (78 mM h^{-1}) [33], and could be improved by increasing the reaction temperature or the amount of biocatalyst used. However, a deeper economic analysis would be needed to confirm whether increasing productivity at the expense of altering some variables might compromise the feasibility of the bioprocess. In any case, these results show that EO-proROL stands as a promising biocatalyst for industrial production of natural isoamyl butyrate.

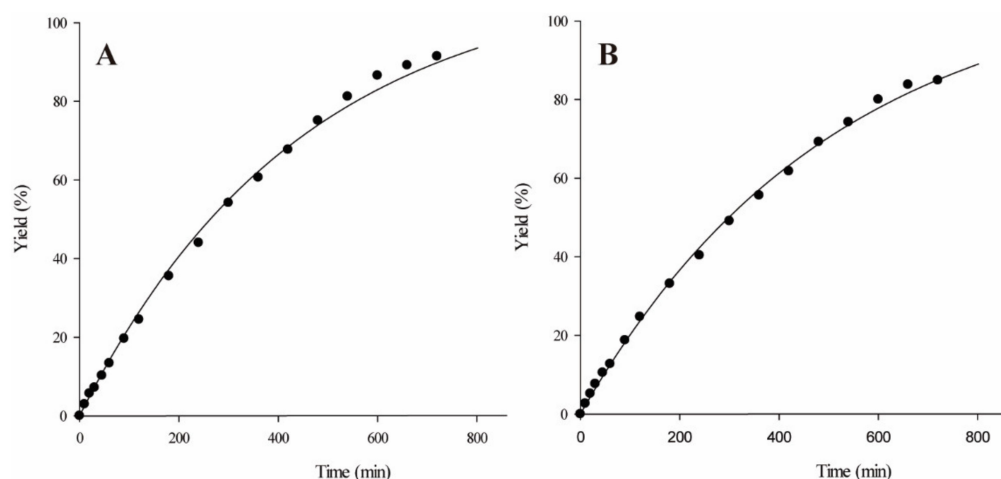


Figure 4. Isoamyl butyrate esterification yield profile with butyric acid and commercial isoamyl alcohol (A) or isoamyl alcohol contained in fusel oil (B) as substrate. The solid line corresponds to the quadratic fitting of the experimental data.

The initial esterification rates were close to $5.4 \mu\text{M min}^{-1}$ with both alcohols (Figure 5), which suggests that the biocatalyst reacted identically with isoamyl alcohol present in both substrates. Operational stability was also identical with both substrates (Figure 5). Thus, the relative yield decreased by about 40% after 5 reaction cycles with both. Similar operational stability results have been reported in ethyl butyrate [57] and butyl acetate production [49].

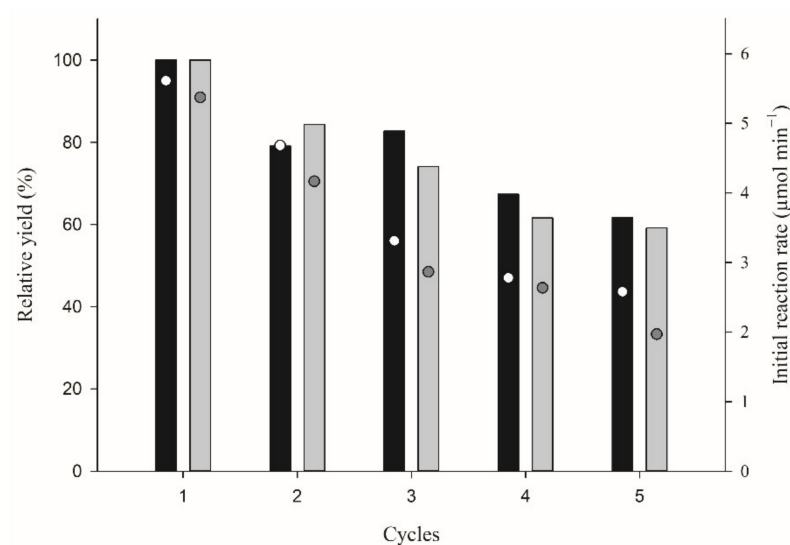


Figure 5. Relative yield (%) of consecutive isoamyl butyrate esterification cycles with butyric acid and commercial isoamyl alcohol (black) or isoamyl alcohol contained in fusel oil (light grey) as substrate. The yield for the first cycle was taken to be 100%. Initial esterification rate of butyric acid by commercial isoamyl alcohol (white circles) or isoamyl alcohol contained in fusel oil (grey circles).

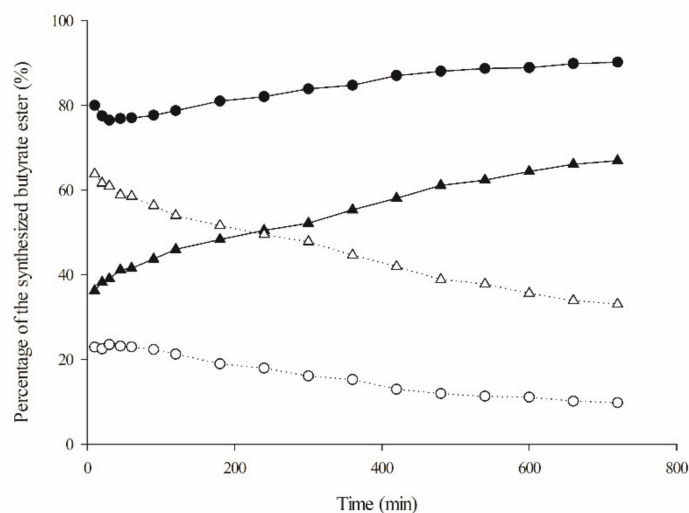
The half-life ($t_{1/2}$) of the biocatalyst during esterification was calculated by fitting the relative yields to Equation (5) (Table 2). Although relative yields were graphically similar with both substrates (Figure 5), the biocatalyst exhibited a slightly higher operational stability with commercial isoamyl alcohol. As a result, the biocatalyst half-life—calculated with Equation (5) described in Section 3.5.2—with the latter (7 batches, equivalent to 84 h reaction) was only slightly greater than that with fusel oil (6 batches or 72 h reaction). This result can be ascribed to the presence of detrimental compounds (e.g., acids, esters, remaining water after desiccation or other impurities) in fusel oil [58].

Table 2. Parameter values for Equation (5) and correlation (R^2) upon fitting of relative yields with commercial isoamyl alcohol and isoamyl alcohol contained in fusel oil.

Alcohol	k_1	k_2	c	R^2
Commercial isoamyl alcohol	2.498	0.0926	99.986	0.9571
Isoamyl alcohol (Fusel oil)	0.1122	0.9124	26.629	0.9944

2.4. Structural Isomers: 3-Methylbutanol and 2-Methylbutanol

As stated in Section 3.7, isoamyl alcohol (3-methylbutanol) and active amyl alcohol (2-methylbutanol) are structural isomers that cannot be fully resolved by ordinary GC [56]. However, using GC/MS here allowed the two to be successfully resolved, thereby allowing us to assess the specificity of EO-proROL for each isomer in the esterification reaction. Commercial isoamyl alcohol was found to consist of 94% 3-methylbutanol and 6% 2-methylbutanol, whereas in fusel oil the proportion was 70% 3-methylbutanol and 30% 2-methylbutanol. Therefore, 2-methylbutanol was the minor isomer in both substrates, albeit in a different proportion. As a result, if EO-proROL had been identically specific for both structural isomers, the esterification products should have retained the isomer composition of the initial alcohols. However, as can be seen from Figure 6, the proportion of 2-methylbutanol ester exceeded the expected value from the very beginning of the reaction (especially with the reaction with fusel oil, which gave 2-methylbutyl butyrate as the major ester). Therefore, EO-proROL was clearly more specific to 2-methylbutanol than it was to 3-methylbutanol, which contradicts the results of previous studies suggesting that enzyme activity was adversely affected by proximity of the methyl group to the hydroxyl group [32,59].

**Figure 6.** Percent composition of the butyrate esters obtained from 3-methylbutanol (solid line, black symbols) and 2-methylbutanol (dotted line, white symbols) in commercial isoamyl alcohol (circles) and fusel oil (triangles).

3. Materials and Methods

3.1. Chemicals

Butyric acid, isoamyl alcohol, acetic acid, fusel oil, a by-product of alcoholic fermentation containing higher alcohols, mainly isoamyl alcohol, pentanol and residual ethanol [34] and isoamyl butyrate, were kindly provided by Hausmann, S.L. (Barcelona, Spain). Poly-methacrylate matrix support D6308 containing epoxy and octadecyl surface groups was complementarily supplied by Purolite® (King of Prussia, PA, USA). Cyclohexane and hexane were purchased from Panreac (Barcelona, Spain). Isoamyl acetate, 3-Å molecular sieves, *p*-nitrophenyl butyrate (*p*NPB) and all non-stated reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Lipase Production and Downstream Processing

Rhizopus oryzae lipase with 28 C-terminal amino acids of its pro-sequence fused to the N-terminal of the mature sequence (proROL) was heterologously expressed in *Komagataella phaffii* (*Pichia pastoris*) under the constitutive promoter P_{GAP} as described elsewhere [60]. Fed-batch runs were followed by removal of biomass and concentration of the enzyme, obtaining a biocatalyst with a specific activity of 70 AU mg^{−1} protein [57].

3.3. Lipase Activity

Lipase activity was determined on a Specord 200 Plus spectrophotometer from Analytic Jena (Jena, Germany), using *p*-nitrophenyl butyrate (*p*NPB) and a procedure adapted from one described elsewhere [61]. Briefly, 500 µL of sample were mixed with 800 µL of reaction buffer to monitor enzyme activity at 348 nm at 30 °C.

3.4. Lipase Immobilisation

Purolite® polymethacrylate matrix support D6308 with epoxide and octadecyl groups (EO) was pre-treated and used to immobilise proROL as described elsewhere [60,62]. The resulting biocatalyst (EO-proROL) was vacuum-filtered, dried on silica gel and stored at −20 °C until use. The specific activity of the immobilised biocatalyst—maintained at 0.35 AU mg^{−1} for comparison purposes during the whole study—was calculated as follows:

$$\text{Specific activity (AU/mg)} = (\text{Final blank act.} - \text{Final supernatant act.}) / \text{dry weight of biocatalyst} \quad (3)$$

3.5. Esterification Reactions

The studied acids (butyric and acetic) were esterified with isoamyl alcohol in the presence of 35 AU of EO-proROL in 15 mL tubes at 30 °C under orbital stirring at 1200 rpm in a Digital Heating Shaking Drybath from Thermo Fisher Scientific (Waltham, MA, USA). All solvents and substrates were dried with 3 Å molecular sieves, and biocatalysts in a drier containing silica gel for 24 h prior to use.

The solvents initially used were cyclohexane and hexane. An acid:alcohol mole *ratio* of 1:1, a butyric acid concentration of 100 mM and a reaction time of 5 h were used for isoamyl butyrate synthesis, and a *ratio* of 1:8, an acetic concentration of 50 mM and a reaction time of 24 h for isoamyl acetate synthesis. The final reaction volume was always 1.6 mL and commercial isoamyl alcohol was used as alcoholic substrate. Reaction yield (%) was calculated by dividing the analysed ester concentration (Section 3.7) by the theoretical maximum ester concentration in each condition.

The operational stability of the biocatalyst was evaluated by allowing it to deposit in the tube bottom and removing depleted medium after the reaction. Then, the biocatalyst was washed three times with solvent and prepared for subsequent esterification.

3.5.1. Influence of the Acid Concentration and Acid:Alcohol Mole Ratio

The influence of the initial acid concentration and acid:alcohol mole *ratio* on isoamyl butyrate biosynthesis was assessed by using Response Surface Methodology (RSM) with production in the first reaction batch and cumulative production after 5 cycles, both in micromoles, as Design of Experiment (DoE) responses. An experimental design of the Box-Hunter type was used with $\alpha = 1.41$ and 3 central points for replication (Table 1). Single-batch and cumulative production of isoamyl butyrate were examined at butyric acid concentrations from 10 to 750 mM and acid:alcohol mole *ratio* from 0.5 to 2 [46,57]. All other reaction conditions were set as described in Section 3.5.

The results obtained for each response were fitted to the following second-order polynomial equation by least-squares regression:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 \quad (4)$$

where Y is the dependent variable (single-batch or cumulative ester production, µmol); X_1 and X_2 are independent variables (initial acid concentration in mM, and acid:alcohol

mole *ratio*); β_0 is an intercept term; β_1 and β_2 are linear coefficients; β_{12} is the interaction coefficient; and β_{11} , β_{22} are quadratic coefficients [30,46,63].

Experimental data were processed with the software Design Expert v. 6.0.8 (Stat-Ease, Inc., Minneapolis, MN, USA) and analysed statistically with SigmaPlot v. 14 (Systat Software, Inc., Chicago, IL, USA).

3.5.2. Reaction Scale Up

The previously established optimal experimental conditions (*viz.*, initial acid concentration and acid:alcohol mole *ratio*) were used to scale up the esterification reaction to a laboratory reactor using a final working volume of 150 mL and mechanical stirring at 500 rpm. The temperature was kept at 30 °C by means of an external jacket.

The initial reaction rate was determined by withdrawing samples at regular intervals during the first 2 h of reaction. Enzyme operational stability was assessed identically as previously in 15 mL tubes. The relative yields of consecutive esterification cycles, as calculated by taking the final yield of the first to be 100%, were used to fit the experimental results to the following two-component first-order exponential decay equation [60,64,65]:

$$Y(\%)_t = 100e^{-k_1 t} + ce^{-k_2 t} \quad (5)$$

where k_1 and k_2 are deactivation coefficients.

3.6. Statistical Analysis

The goodness of fit of the response surfaces to Equation (4) was assessed in terms of R^2 , adjusted- R^2 and predicted- R^2 . An analysis of variance (ANOVA) F -test was used to assess the significance of the obtained equations, their individual coefficients being evaluated with a t -test. The lack of fit (LOF) test was used to assess differences between experimental and pure error in the fitted equations. LOF was calculated by dividing the variation between the actual measurements and the values predicted by the model by the pure error (the variation among any replicates). Any p -values < 0.05 were taken to be statistically significant.

3.7. Gas Chromatography/Mass Spectrometry Analysis

Isoamyl alcohol, isoamyl acetate and isoamyl butyrate were quantified in a GC8860/M S5977E gas chromatograph/mass spectrometer from Agilent (Santa Clara, CA, USA) equipped with a HP-5MS capillary column (30 m $\times$ 250 μ m, 0.25 μ m film thickness). The column temperature was raised from 60 to 112 °C at 3 °C min^{−1} and then further raised to 246 °C at 12 °C min^{−1}, the final level being held for 5 min. The injected sample volume was 1 μ L and the split *ratio* 50:1. Helium at a constant flow-rate of 1 mL min^{−1} was used as carrier gas. The inlet and mass transfer line temperatures were 230 and 250 °C, respectively, and the ion source and quadrupole temperatures were set at 230 and 150 °C, respectively. Mass spectra were acquired over the m/z range 35–350 after 1.6 min of solvent delay. Because most reported chromatographic methods fail to fully resolve the structural isomers of isoamyl alcohol and active amyl alcohol, they were referred to as “(iso)amyl alcohol” and “(iso)amyl esters” for comparison unless stated otherwise [56].

4. Conclusions

Immobilised *Rhizopus oryzae* lipase (ROL) proved a suitable biocatalyst for producing natural isoamyl esters, especially in cyclohexane. A central composite rotatable Box-Hunter design predicted a 410 mM initial butyric acid concentration and an acid:alcohol mole *ratio* of 0.5 to be the optimum values for maximising single-batch and cumulative production of esters, which peaked at 256.28 and 1282.78 μ mol, respectively. These predictions were validated by deviations less than 2.5% from the experimental results. The reaction was successfully scaled up from 15 mL tubes with orbital stirring to a laboratory bioreactor with a final volume of 150 mL and mechanical stirring. The results obtained by using commercial isoamyl alcohol in the bioreactor were compared with those provided by isoamyl alcohol contained in fusel oil. Both substrates gave similar yields (91% with commercial alcohol

vs. 84% with isoamyl alcohol contained in fusel oil), initial reaction rate ($5.4 \mu\text{M min}^{-1}$ with both substrates), operational stability (40% activity loss after 5 runs with both) and productivity (31.09 vs. 28.7 mM h^{-1}). The enzyme was more specific to 2-methylbutanol than it was to 3-methylbutanol.

Based on the results, EO-proROL stands as a suitable biocatalyst for industrial production of natural isoamyl butyrate, even from an inexpensive raw material, such as fusel oil, to comply with the principles of circular economy.

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